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ARTICLE

Patterns of folliculogenesis in ducks following the administration of a gonadotropin-releasing hormone 1 (GnRH) analogue

Khairy M.A. Zoheir [a,b,*], **R.G. Ahmed** [c]

[a] *King Saud University, College of Pharmacy, Riyadh, Saudi Arabia*
[b] *National Research Centre, Cell Biology Department, Dokki, Cairo, Egypt*
[c] *Division of Anatomy and Embryology, Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt*

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Abstract The efficacy of synthetic gonadotrophin-releasing hormone (GnRH) analogue in improving the folliculogenesis of ducks has not been established. The aim of the study was to investigate the effect of oral administration of GnRH analogue as luteinizing hormone releasing hormone A_2 (LHRH-A_2) on expression of relevant genes, egg production, changes of hormone levels and an ovarian follicle development in ducks. Five hundred ducks at 220 days old were randomly allotted to five groups, where each bird received daily in food 0, 5, 10, 15, or 20 μg LHRH-A_2 for 60 days. In all treated groups, a non-significant increase in the level of GnRH receptor was noticed as compared to the corresponding control. Interestingly, the egg product in the 10 and 15 μg LHRH-A_2 groups was profoundly increased ($P < 0.05$) if compared to 0 and 5 μg LHRH-A_2 groups or control. A reduction in circulating prolactin (PRL) levels occurs concurrently with an increase in progesterone (P_4) and estradiol (E_2) particularly in 5, 10 and 15 μg LHRH-A_2 groups. Maximal apoptotic percentage for the granulosa cells was obtained in 20 μg LHRH-A_2 group as compared to control or 5, 10 and 15 μg treatment groups. Finally, these data suggest that the oral administration of 10 and 15 μg LHRH-A_2 may induce ovarian cycle and play vital gonadotrope

* Corresponding author at: King Saud University, College of Pharmacy, Riyadh, Saudi Arabia.
E-mail addresses: khma25@gmail.com, kzoheir@ksu.edu.sa (K.M.A. Zoheir), ragabb900@yahoo.com (R.G. Ahmed).

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role during the folliculogenesis process in ducks. This study also demonstrated a need to concentrate further research on the potential effect of GnRH during the early period to improve the reproductive performance.

1. Introduction

The natural gonadotropin-releasing hormone (GnRH) is a deca-peptide produced by specialized neurons in the basal hypothalamus and released to the pituitary via a portal circulatory system [10,61]. Its ability to stimulate luteinizing hormone (LH) and follicle stimulating hormone (FSH) release has been utilized for various veterinary purposes, including regulation of ovarian function and optimization of timed insemination in controlled breeding programs [62], induction of ovulation in the post-partum period [44], and treatment of ovarian follicular cysts in cattle [36]. Also, it is well known that GnRH is involved in the regulation of ovarian development in mammals through paracrine/autocrine pathways [37,50,53]. GnRHs have been isolated and sequenced from vertebrate species [66] (Wierman, 1996) across the phylogenetic scale including tunicates, primitive protochordates [47]. In several species, more than one form of GnRH exists [47].

Because the GnRH family plays an important role in normal reproduction, interest has centered on developing more potent GnRH analogues (both agonists and subsequently antagonists) to intervene in some diseases of the reproductive system. The birds' embryos can be used as a model for investigating toxicity of different compounds such as estrogenic compounds because they are prove to be easy to access [4,16]. Authors have been described how to investigate the effect of environmental or synthetic estrogenic compounds using direct injection into eggs in the early stages of incubation [4,16]. Two types of GnRH, GnRH-I and GnRH-II are secreted from avian hypothalamus. GnRH-I plays a critical role in the reproduction in birds [35]. Furthermore, GnRH is required for a normal reproduction in vertebrates, and released in pastille fashion from neurosecretory cells in the hypothalamus [72]. During assisted reproductive technology protocols, GnRH agonists are often used to deliver pulsatile release of GnRH, which in turn, increases the release of FSH and LH in cases of hypogonadotropic hypogonadism [54]. It has suggested that the use of GnRH agonists for the control of reproduction is of growing interest to increase the efficiency of reproductive performances in farm animals [25,41]. The use of GnRH and its analogue has been investigated in cows [59,61], mare [21], sheep and goats [26], fish [68], dogs [8], wildlife [2,5,42,54] and poultry [37,48]. Although parenteral administration of GnRH agonists practiced in flocks is difficult, GnRH agonists were primarily used by parenteral administration as discussed by [26,48]. Interestingly, GnRH family plays an important role in normal reproduction, so it has centered on developing more potent GnRH analogues (both agonists and subsequently antagonists) to intervene in some diseases of the reproductive system [25].

However, studies on GnRH-I expression are largely confined to the hypothalamus, with none on avian ovary. Although the prepubertal changes of hypothalamic GnRH-I have been documented in birds [12], nothing is known about the pattern of ovarian expression of GnRH-I during this period of posthatch development. Thus, the objectives of this study were to understand the effect of oral administration of Gonadotropin-releasing hormone (GnRH) analogue as Luteinizing Hormone Releasing Hormone A_2 (LHRH-A_2) on expression of relevant genes, changes of hormone levels, egg production and ovarian follicle development in ducks.

2. Materials and methods

2.1. Animals and treatments

Five hundred Pekin ducks (*Anas platyrhynchos*) (one male for every three females), which were hatched and held in same conditions, were allotted to five treatment groups of 100 birds each. The feeding procedure during the whole experiment followed the feeding and housing standards set for this breed. Ducks were given free access to water and food under the same photoperiod of 12 h, which consisted of natural illumination during the daytime, plus supplementary illumination of 80 lux by fluorescent tubes at times after sunset and before sunrise. At age 220 days, ducks received 0, 5, 10, 15, or 20 μg of LHRH-A_2 daily mixed with feed. Experiments continued for 60 days.

2.2. Egg production and egg quality

Eggs were collected daily and egg production was recorded. Every treatment group contained five replicates and each replicate contained 20 birds. Mean egg weight/replicate was determined by weighing all the eggs on three consecutive days every week. The yolk, albumin, and shell percentages were determined using five eggs/pen at a biweekly interval.

2.3. Detection of granulosa cell apoptosis

At the end of the experiment, three ducks of each replicate were sacrificed. The ovaries were immediately removed by cervical dislocation, and kept in PBS solution. The granulosa layers were then collected from the follicle wall according to the technique described by [20], and then the cells were dissociated in collagenase containing medium PBS. Cells were stained with Annexin V, a phospholipid binding protein that detects early apoptosis, and with propidium iodide (PI), a membrane impermeable stain, to discriminate between dead and apoptotic cells. Briefly, the Annexin V incubation reagent was prepared by mixing 10 μL of 10× binding buffer, 10 μL of PI, 1 μL of Annexin V-FITC, and 79 μL of distilled water. The cells were gently re-suspended in the Annexin V incubation reagent in the dark for 15 min at 18–24 °C. Finally, 400 μL of 1× binding buffer was added to each sample. After staining, cells were analyzed by Flow Cytometry within 1 h for maximal signal. Vital cells are negative for both PI and Annexin V, apoptotic cells are Annexin V positive and PI negative, while necrotic cells are positive for both PI and Annexin V.

2.4. Determination of serum biochemical parameters and hormone level

Blood samples (were randomly selected from each replicate) were taken from the brachial wing vein at 40 and 60 days at 8 h, after fasting for 18 h. Blood samples were allowed to clot, then centrifuged at 3000 rpm for 15 min to collect blood serum. An aliquot of serum was stored at 20 °C until assayed for progesterone (P_4), prolactin (PRL) and estradiol (E_2). E_2, P_4 and PRL levels were assayed using the commercial RIA-test kit. The assay sensitivity was less than 5 pg/ml for both E_2 and P_4 and was less than 1.0 ng/mL for PRL. Coefficients of variations between assays in E_2 and P_4 were less than 10%, and within assay was less than 7% while were less than 6.0–9.7%, and within assay was less than 2.2–5.4% for PRL.

2.5. RNA extraction

Total RNA was extracted from the hypothalamic tissue using Trizol and the RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer. Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose–formaldehyde gel to verify their integrity.

2.6. Reverse transcription (RT) and polymerase chain reaction (PCR)

Two micrograms of total RNA was reverse transcribed by incubation at 42 °C for 1 h in a 20 µl mixture consisting of 10 U avian myeloblastosis virus reverse transcriptase, 20 U RNase inhibitor, 2.4 µM Oligo (dT10) primer, 1 mM each dNTP, 50 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 10 mM DDT, 0.5 mM spermidine. RNA samples were denatured at 80 °C for 5 min and placed on ice for 5 min together with the Oligo (dT10) primer and dNTP before reverse transcription (RT). The RT reaction was terminated by heating at 95 °C for 5 min and quickly cooled on ice. 2 µL of RT reaction mix was used for PCR in a final volume of 50 µl containing 1 U Taq DNA polymerase, 5 mM Tris–HCl (pH 9.0), 10 mM NaCl, 0.1 mM DDT, 0.01 mM EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mM each dNTP, 1.0–2.0 mM $MgCl_2$, 0.5 µM specific primers for respective target genes. The PCR primers for GnRH, and β-actin were designed according to [72]. The nucleotide sequences of these primers and the PCR conditions set for respective genes were shown in Table 1. Each target gene was co amplified with β-actin in the same reaction. By adjusting the ratio of β-actin primers to primers of the target genes in the reaction system, the overall PCR amplification efficiency of β-actin can be reduced to the level comparable to that of target genes. Different controls were set to monitor the possible contaminations of genomic and environment DNA both at the stage of RT and PCR. All samples were included in the same run of RT-PCR and repeated three times. Both RT and PCR were performed in a Gene Amp PCR System 9600.

Table 1 Showing primer sequences of both GnRh and β-actin.

Target genes	PCR products	Primer sequences
GnRH	202 bp	F: 5′-GCTTGGCTCAACACTGGTCT-3′ R: 5′-CTGGCTTCTCCTTCGATCAG-3′
β-Actin	282 bp	F: 5′-ACGTCGCACTGGATTTCGAG-3′ R: 5′-TGTCAGCAATGCCAGGGTAC-3′

2.7. Data analysis

Data are expressed as means ± standard error (SE), and were evaluated for statistically significant difference by one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test, using Statistical Analysis System Package for *P*-values <0.05 were considered significant.

3. Results

3.1. Effect of gonadotropin-releasing hormone on mRNA expression of GnRH

Data obtained about the expression of gonadotropin-releasing hormone (GnRH) receptor of control and treated groups were shown in Table 1. In all treated groups, the elevation in the expression level was non-significant (LSD; $P > 0.05$) when compared to respective control values (1.00, 1.10, 1.15 and 1.15 ± 0.03 and 0.95 ± 0.04) (Fig. 1).

3.2. The production performance of ducks

It is clear from Table 2 that the egg production for each bird in the 10 and 15 µg LHRH-A_2 groups increased significantly ($P < 0.05$) compared to the 0 and 5 µg LHRH-A_2 groups even though this production was suppressed when the dose increased to 20 µg. Furthermore, no difference in the egg weight, egg shape index, egg shell thickness, albumen percentage, yolk percentage and egg shell percentage was recorded between the control and different treatment groups (Table 2).

3.3. The apoptosis of follicle granulosa cells

The results of apoptotic rate in different groups were represented in Table 3. The apoptotic percentage for granulosa cells was enormously increased ($P < 0.05$) in 20 µg LHRH-A_2 only compared to 5, 10, and 15 µg treatments or control group.

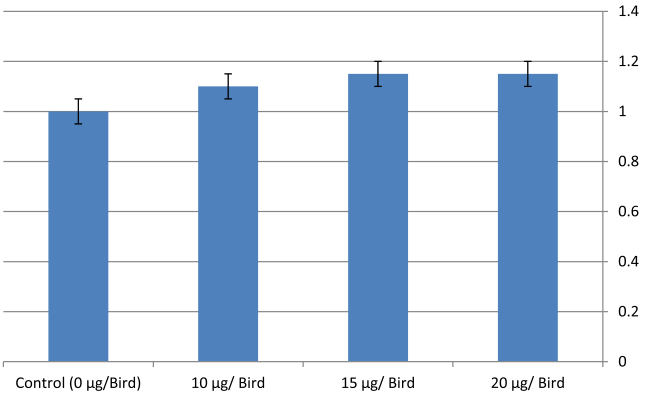

Figure 1 Histogram showing GnRH gene expression in ducks hypothalamic tissue in each treatment group.

Table 2 The production performance of ducks in each treatment group.

Treatment (μg/bird)	Mean daily egg production	Egg weight (g)	Egg shape index	Eggshell thickness (mm)	Percentage of egg weight		
					Albumen	Yolk	Eggshell
5	0.33 ± 0.01[b]	150.0 ± 8.0	1.27 ± 0.1	0.54 ± 0.0	55.9 ± 1.6	35.20 ± 1.0	11.9 ± 0.3
10	0.38 ± 0.01[a]	142.0 ± 6.0	1.30 ± 0.0	0.51 ± 0.0	55.8 ± 1.8	36.2 ± 0.6	12.9 ± 1.2
15	0.38 ± 0.01[a]	148.7 ± 4.4	1.2 ± 0.05	0.55 ± 0.0	58.2 ± 1.56	33.9 ± 1.0	11.9 ± 0.7
20	0.25 ± 0.01[c]	145.7 ± 1.2	1.29 ± 0.0	0.56 ± 0.0	53.3 ± 1.39	34.2 ± 1.3	10.2 ± 0.5
Controls	0.31 ± 0.02[b]	144.5 ± 2.0	1.28 ± 0.0	0.6 ± 0.01	52.5 ± 1.7	32.7 ± 0.6	11.1 ± 1.0

Values within the same line with different superscripts are significantly different at $P < 0.05$.

Table 3 The apoptosis of granulose cell in ducks follicles in each treatment group.

	Treatments (μg/bird)				
	0	5	10	15	20
Apoptosis (%)	8.80 ± 2.60[ab]	5.89 ± 1.68[b]	5.95 ± 1.31[b]	5.49 ± 1.12[b]	13.03 ± 1.29[a]

Values within the same line with different superscripts are significantly different at $P < 0.05$.

Table 4 Serum hormone parameters of ducks in each treatment group.

	Treatments (μg/bird)				
	0	5	10	15	20
PRL (ng/mL)	0.33 ± 0.05[a]	0.18 ± 0.02[b]	0.12 ± 0.01[c]	0.10 ± 0.02[c]	0.30 ± 0.04[a]
P (ng/mL)	5.14 ± 0.26[bc]	6.31 ± 1.01[ab]	7.8 ± 1.02[a]	7.34 ± 0.95[a]	3.95 ± 1.45[c]
E2 (pg/mL)	199.59 ± 102.60[b]	275.96 ± 74.76[ab]	387.1 ± 2.1[a]	371.1 ± 42.4[a]	182.20 ± 87.03[b]

Values within the same line with different superscripts are significantly different at $P < 0.05$.

3.4. Serum hormone levels

As indicated in Table 4, the levels of progesterone (P_4) and estradiol (E_2) increased significantly ($P < 0.05$) in 5, 10 and 15 μg treatment groups as compared with the 20 μg treatment group or corresponding control (Table 4). However, the opposite direction was observed in the level of prolactin (PRL) (Table 4).

4. Discussion

A gonadotropin-releasing hormone analogue (GnRH analogue or analog), also known as a luteinizing hormone releasing hormone agonist (LHRH agonist) or LHRH analogue is a synthetic peptide drug modeled after the human hypothalamic GnRH. A GnRH analogue is designed to interact with the GnRH receptor and modify the release of pituitary gonadotropins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)] for therapeutic purposes. A GnRH agonist is an analogue that activates the GnRH receptor resulting in increased secretion of FSH and LH. In the current study, a non-significant increase in the level of GnRH receptor was observed in all treated groups if compared to the control. Similarly, through radioligand binding assays, ligand-specific binding sites for GnRH were demonstrated on granulosa and luteal cells [22,45] and on Leydig cells [28,57]. These findings were further confirmed in subsequent studies by other researchers [27,65]. In situ hybridization studies revealed the localization of GnRH mRNA in granulosa cells of primary, secondary, and tertiary follicles in the ovary [7,65]. Presence of mRNA for GnRH receptors has also been identified in human granulosa luteal cells (hGLCs) using reverse transcription polymerase chain reaction (RT-PCR) techniques [23,39]. In addition, the presence of GnRH receptor mRNA expressions was recorded in bovine ovary [49]. RT-PCR studies revealed the presence of GnRH-R mRNA expression in both follicles and corpus leteum (CL) tissues. On the other hand, Ni Y et al. [38] have demonstrated the changes of GnRH-I, POMC and NPY mRNA transcription in hypothalamus and IGF-I and leptin levels in serum of Shaoxing ducks during puberty. Their results have indicated that the down-regulation of POMC and NPY genes in hypothalamus coincides with the up-regulation of GnRH-I gene to initiate sexual maturation in ducks. According to Ni et al. [38] who demonstrated that, the developmental pattern of GnRH-I mRNA expression reflects a slow maturational process of the GnRH neurons during maturation in birds. However, the biosynthetic mechanism(s) responsible for developmental changes in GnRH-I mRNA levels is (are) currently unknown. In addition and according to study of Zhao et al. [72] who concluded that activation of hypothalamic expression of GnRH mRNA and elevated level of serum E_2 contribute to enhanced laying rate. Taking the previous information, it can be concluded that the GnRH receptor is expressed in the majority of gonadotropes suggesting its role in the regulation of gonadotropin secretion. This has led to the suggested hypothesis that GnRH has spe-

cific role in the regulation of the gonadotrope function in ducks.

In the present study, in 10 and 15 μg luteinizing hormone releasing hormone A_2 (LHRH-A_2) groups, the egg product was increased significantly ($P < 0.05$) compared to 0, 5 and 20 μg LHRH-A_2 groups or control. However, the treatments had little effect on the index of egg quality. Concomitantly with this finding, the maximal apoptotic percentage for the granulosa cells was observed in 20 μg LHRH-A_2 group as compared to control or 5, 10 and 15 μg LHRH-A_2 groups. Parenteral administration of GnRH agonists can stimulate ovulation in many vertebrates [1,26,48] and increased the number of large preovulatory ovarian follicles [6]. It was clearly that GnRH could induce ovulation of follicles that were physiologically immature (< 11 mm) [43,61]. In laying broiler breeder hens, increasing GnRH release can result in increased plasma LH and decreased FSH, and, in turn, increase the number of large preovulatory ovarian follicles [6]. Interestingly, an uninterrupted subcutaneous administration of a GnRH-agonist for 2 weeks also rapidly induced a normal estrus, with spontaneously occurring gonadotrophin surges and fertile ovulation [8,9]. GnRH treatment appears to be effective in stimulating a new ovulation/luteinisation of another follicle as it would in a non-cystic cow [18,24]. On the other hand, the apoptosis rate of granulosa cells was a predictor of the developmental potential of the corresponding oocytes [58]. In human luteinized granulosa cells, GnRH analogue triggered a dose dependent increase in the incidence of apoptosis [24,58]. Moreover, Zhao et al. (2000) demonstrated that GnRH-A-induced increased number of apoptotic bodies in human granulosa cells (obtained during oocyte retrieval for in vitro fertilization). Whitelaw et al. [65] reported that during follicular phase, attretic follicle in rat showed a high mRNA expression for GnRH receptors. This variation with our experiment might be attributed to numerous factors such as species, environment, management, breed, animal age, breeding season, individual farm effects, interval from calving to first service, reproductive/lactational status, and type of breeding. Generally, the current administration of LHRH-A_2 can significantly influence apoptosis of follicle granulose cells of ducks where the physiological doses of LHRH-A_2 can inhibit granulosa cell apoptosis of the follicle, and higher does could induce follicular granulosa cell apoptosis. These observations may be reinforced by several investigators [29,55,69,70]. Furthermore, the influence of LHRH-A_2 on ovary granulosa cells maybe associated with the different pathways. There are two possible channels by which LHRH-A_2 may modulate the function of the ovary. First, LHRH-A_2 acts on GnRH receptors in the pituitary to regulate the production and release of gonadotropins FSH and LH from the anterior pituitary [60]. Secondly, LHRH-A_2 acts on the ovary directly. The direct effects of GnRH and its agonist on the ovary have been supported by the demonstration of specific, high-affinity binding sites for GnRH and its agonist [58,51]. It can be inferred from the above mentioned results that LHRH-A_2 (10 and 15 μg) is involved in the regulation of follicular development, increase egg production and decrease apoptotic rate in ducks.

Worth noting in the current study is that the concentrations progesterone (P_4) and estradiol (E_2) were markedly increased ($P < 0.05$) in 5, 10 and 15 μg LHRH-A_2 groups as compared to the 20 μg LHRH-A_2 and control groups while the opposite pattern was noticed in the level of prolactin (PRL). Similarly, Beck et al. [3] and Khan et al. [26] showed that treatment with GnRH resulted in higher plasma progesterone concentrations. In addition, GnRH can increase serum P_4 concentrations in the cow [19,67]. This increased progesterone could enhance uterine function leading to increased conceptus growth/development and production of the antiluteolytic embryonic protein interferon-tau, which suppresses prostaglandin $F_{2\alpha}$ release. Moreover, it has been shown that high levels of progesterone during the luteal phase delay the process of luteolysis [26,30,40]. Foster et al. [14] and Howard et al. [19] reported that the administration of GnRH on days 5 or 6 of the estrous cycle overrides the negative feedback of progesterone on the anterior pituitary, thereby allowing the secretion of both LH and FSH, resulting in ovulation or luteinization of the follicle, and subsequent formation of a corps leutum CL. Therefore, the increase in CL number is likely responsible for the increased progesterone concentrations in the GnRH group. Also, studies on sheep [33] (McNatty and Land, 1979), mouse [11], humans [32], porcine [63], chicken and domestic hen [52] and turkey [46] suggest that low concentration of prolactin is required for synthesis of progesterone, since high concentration of prolactin decreases progesterone levels by stimulating hydroxysteroid dehydrogenase enzyme, which brings about the catabolism of progesterone [52,64]. Similar observation in birds is not clear but prolactin inhibits granulose cell progesterone at high doses [56] but not at lower, physiological doses [17]. These support our findings that low levels of progesterone as observed in ducks of the control group, which may also be due to the catabolism of progesterone to 5α-dihydroprogesterone or its epimer 5β-dihydroprogesterone. Marrone et al. [31] and Reddy et al. [52], have been brought about by increased peripheral prolactin concentration. On the other hand, estradiol in conjunction with progesterone primes the release of LH [15]. The increase in oestradiol-17β levels after inhibiting the high levels of prolactin suggests that prolactin levels may be interfering with oestradiol synthesis [52]. Such observations in birds are not clear, but it has been shown that prolactin can inhibit only LH-stimulated oestradiol-17β production by chicken follicles of < 1 mm diameter [71]. High prolactin levels may either interfere with the synthesis of androgens or FSH-induced aromatase activity, leading to oestradiol-17β formation [52,53]. It is presumed that among the treated birds, the increase in oestradiol could have been due to decreased prolactin concentration. Since high levels of prolactin may inhibit ovarian follicular steroidogenesis, not only does this interfere with aromatase but it also reduces the production of androgen precursor necessary for oestradiol-17β production by the theca cells [34] and androstenedione is thecal in origin and its release is stimulated by LH. It is possible that decreased prolactin levels are attributed to increase in oestradiol and progesterone levels, which may act as a positive feedback on secretion of LH and subsequent ovulation [13,52]. It was found that the higher egg production was associated with positively correlated responses of high concentrations of LH, E_2, and P4 required for completion of egg formation [52]. It is observed from the above mentioned results that decreasing the prolactin levels with GnRH treatment resulted in an increase in egg production which could be achieved by abolishing the negative effect of prolactin on gonadotrophins steroidogenesis in the gonads, activating the gonadotrophin receptors on the gonads, increasing the levels of oestradiol and progesterone concentrations, which in turn feed back on the anterior pituitary for elic-

iting the LH surge for ovulation and oviposition and increased egg production with a decrease in intersequence pause days.

5. Conclusion

The results suggest that LHRH-A_2 is involved in the regulation of follicular development and increase egg production in ducks. Also, the results demonstrate that LHRH-A_2 may play an important gonadotrope function during the folliculogenesis process in ducks. Interestingly, a dose of 10 or 15 μg LHRH-A_2 mixed with feed/bird/day may be the optimal dosage. Further studies on optimization of the GnRH dosage, schedule, and possible secondary consequences of the presence of GnRH in the egg yolk are needed. Furthermore, such an approach and procedure could be extended to other species of economic importance such as chicken, geese, and quails.

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